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(54) Title: PIGGYBAC TRANSFORMATION SYSTEM

(57) Abstract: The present invention is directed to a transformation system for making transgenic organisms that includes a vector containing a modified *piggyBac* transposon into which is inserted an enhanced green fluorescent protein gene linked to a polyubiquitin promoter sequence and a nuclear localizing sequence; and a helper transposase vector that includes an *hsp70* promoter sequence upstream of the putative *piggyBac* promoter that increases the transformation frequency of this system.

PIGGYBAC TRANSFORMATION SYSTEM

BACKGROUND OF INVENTION

Field of the Invention

This invention relates to a transformation system that includes a gene transfer vector containing a modified *piggyBac* transposon (pB) and having the insertion of a marker construct containing an enhanced green fluorescent protein gene (EGFP) linked to a polyubiquitin promoter gene and a nuclear localizing sequence. The invention further relates to a helper vector containing a heat shock protein gene and to methods for using this system to transform eukaryotic cells as well as transgenic organisms produced using the system, especially insect cells and insects, respectively.

Description of the Related Art

The *piggyBac* transposable element from the cabbage looper moth, *Trichoplusia ni* (Cary et al., Virology, Volume 161, 8-17, 1989) has been shown to be an effective gene-transfer vector in the Mediterranean fruit fly, *Ceratitis capitata* (Handler et al., Proc. Natl. Acad. Sci. USA, Volume 95, 7520-7525, 1998). Use of an unmodified transposase helper under *piggyBac* promoter regulation indicates that *piggyBac* retains autonomous function in the medfly, since transcriptional regulation was maintained, as well as enzymatic activity. This observation was unique since all other successful insect germline transformations had been limited to dipteran species using vectors isolated from the same or another dipteran. The initial transformation of medfly (Loukeris et al., Science, Volume 270, 2002-2005, 1995) used the *Minos* vector from *Drosophila hydei* (Franz & Savakis, Nucl. Acids Res., Volume 19, 6646, 1991), and *Aedes aegypti* has been transformed from *Hermes* (Jasinskiene et al., Proc. Natl. Acad. Sci. USA, Volume 95, 3743-3747, 1998) from *Musca domestica* (Warren et al., Genet. Res. Camb., Volume 64, 87-97, 1994) and *mariner* (Coates et al., Proc. Natl. Acad. Sci. USA, Volume 95, 3748-3751, 1998) from *Drosophila mauritiana* (Jacobson et al., Proc. Natl. Acad. Sci.

USA, Volume 83, 8684-8688, 1986). *Drosophila melanogaster* has been transformed as well by *Hermes* (O=Brochta et al., Insect Biochem. Molec. Biol., Volume 26, 739-753, 1996) *mariner* (Lidholm et al., Genetics, Volume 134, 859-868, 1993), *Minos* (Franz et al., Proc. Natl. Acad. Sci. USA, Volume 91, 4746-4750, 1994) and by the *P* and *hobo* transposons originally discovered in its own genome (Rubin and Spradling, 1989; Blackman et al., EMBO J., Volume 8, 211-217, 1989). *Drosophila virilis* also has been transformed by *hobo* (Lozovskaya et al., Genetics, Volume 143, 365-374, 1995; Gomez & Handler, Insect Mol. Biol., Volume 6, 1-8, 1997) and *mariner* (Lohe et al., Genetics, Volume 143, 365-374, 1996). While the restriction to dipteran vectors is due in part to the limited number of transposon systems available from non-dipteran species, phylogenetic limitations on transposon function is not unexpected considering the deleterious effects functional transposons may have on a host genome. This is, indeed, reflected by the high level of regulation placed on transposon movement among species, among strains within a host species, and even among cell types within an organism (Berg & Howe, *Mobile DNA*, American Society for Microbiology, Washington, D.C. 1989).

The ability of *piggyBac* to function in several dipteran species will be supportive of its use in a wider range of insects, if not other organisms. Most other vector systems function optimally, or have been only tested with their helper transposase under *hsp*70 promoter regulation. The transposition efficiency of most vectors has been also found to be influenced by the amount of internal DNA inserted, the position of this DNA within the vector, and the amount of subterminal DNA remaining in the vectors.

The widespread use of *piggyBac* will be limited by the availability of easily detectable and unambiguous transformant markers. Most Drosophila transformations, as well as the few nondrosophilid transformations reported have depended on transformant selection by rescue of a mutant visible phenotype, usually eye pigmentation (Ashburner et al., Insect Mol. Biol., Volume 7, 201-213, 1998). Unfortunately, most insect species have neither visible mutant strains, nor the cloned DNA for the wild

type allele of the mutation, and these species require use of new dominant-acting marker genes that confer, preferably, a visible phenotype.

The present invention, discussed below, provides a system that includes vectors for transforming eukaryotic cells, derived from *piggyBac* transposons that are different from related art vectors. Furthermore, the present invention increases the transformation frequency by about eight-fold compared to other *piggyBac* transformation systems.

Summary of the Invention

It is therefore an object of the present invention to provide a transformation system contains a vector that includes DNA derived from a *piggyBac* transposon element that allows for the almost precise excision of at least a second DNA sequence that is heterologous and included in the construct and insertion of at least said second heterologous DNA sequence into eukaryotic cells after introduction of the transformation construct containing said first and at least a second DNA into said cell that is then used to form a transgenic organism wherein said transgenic organism is detectable under ultraviolet light.

Another object of the present invention is to provide a transformation system that includes a vector containing a modified *piggyBac* sequence, a sequence for marker expression linked to a polyubiquitin promoter and a nuclear localizing sequence and a helper vector including a heat shock protein gene wherein said system causes an increase in transformation frequency compared to other *piggyBac* transformation systems.

A still further object of the present invention is to provide a vector containing a modified *piggyBac* sequence and an enhanced green fluorescent protein sequence linked to a polyubiquitin promoter and a nuclear localizing sequence.

A still further object of the present invention is to provide a vector that is useful in transforming eukaryotic cells having the sequence SEQ ID No 6.

Another object of the present invention is to provide a transgenic organism that is detectable under ultraviolet light.

A further object of the present invention is to provide a eukaryotic transgenic organism that has been transformed using a transformation system that includes vector containing a modified *piggyBac* sequence, an enhanced green fluorescent protein gene linked to a polyubiquitin promoter and a nuclear localizing sequence, and a helper vector containing a heat shock protein gene promoter.

A still further object of the present invention is to provide a transgenic insect that has been transformed using a vector having the sequence SEQ ID NO 6.

Further objects and advantages of the present invention will become apparent from the following description.

Brief Description of the Drawings

Figure 1(a) is a photograph of eye color phenotypes of *Dm*[pBw] transformants.

Figure 1(b) is a photograph of a w[m] host strain fly (top) and orange-eye *Dm*[pBw,*gfp*] transformant fly (bottom) under brightfield (left) and UV light (right).

Figure 1 (c) is a photograph of a *w*[m] host strain fly (top) and white-eye *Dm*[pBw, gfp] transformant fly (bottom) under brightfield (left) and UV light (right).

Figure 2 (a) is a schematic (not to scale) of the pB[Dmw] vector showing the *Bgl*II, *Sal*I, and *Nsi*I restriction sites used to digest the genomic DNA, and the probes used for hybridization (bars). Above the schematic are distances in kilobases used to calculate internal restriction fragment sizes and minimum sizes for junction fragments. *PiggyBac* vector sequences are shaded gray, and the mini-*white* marker gene is white.

Figure 2 (b) shows an autoradiogram of a Southern DNA hybridization analysis of *Dm*[pBw] transformant sublines, and w[m] host strain control samples from transformants using the pBΔSac (experiment I) or phsp-pBac (experiment II) helpers using *Bgl*II digestion and *Sph/Hpa piggyBac* as probe. DNA size markers are shown to the left of the autoradiogram. M (male) and F (female)

designations refer to G0 lines, with the numbers below referring to their respective G1 sublines.

Figure 2(c) shows a Southern DNA hybridization analysis of *Dm*[pBw] transformant sublines and *w*[m] host strain control samples from transformants, using the pBΔSac (experiment I) or phsp-pBac (experiment II) helpers, using *Sal*I digestion and *Hpa/Ase piggyBac* as probe. DNA size markers are shown to the left of the autoradiogram. M (male) and F (female) designations refer to G0 lines, with the numbers below referring to their respective G1 sublines.

Figure 2 (d) shows a Southern DNA hybridization analysis of *Dm*[pBw] transformant sublines and *w*[m] host strain control samples from transformants, using the pBΔSac (experiment I) or phsp-pBac (experiment II) helpers, using *Nsi*I digestion and *Nsi/Hpa* + *Hpa/Nsi* probes. DNA size markers are shown to the left of the autoradiogram. M (male) and F (female) designations refer to G0 lines, with the numbers below referring to their respective G1 sublines.

Figure 3(a) is a schematic (not to scale) of the pB[Dmw, PUbnlsEGFP] vector showing the *Bgl*II, *Xho*I, and *Pst*I restriction sites used to digest the genomic DNA, and the probes used for hybridization (bars). The *Sph/Hpa piggyBac* as probe contains 0.67 kb of vector sequence (*Sph*I to *Bgl*II) with *Bgl*II to *Hpa*I *piggyBac* sequence deleted from the vector. Above the schematic are distances in kilobases used to calculate internal restriciton fragment sizes and minimum sizes for junction fragments. *PiggyBac* vector sequences are shaded gray, the mini-*white* marker gene is white, and the EGFP marker gene is hatched.

Figure 3(b) is an autoradiogram of a Southern DNA hybridization analysis of *Dm*[pBw, *gfp*] transformant sublines, and wild type (wt) and *w*[m] host strain control samples using *Bgl*II digestion and *Sph/Hpa piggyBac* as probe. DNA size markers are shown to the left of the autoradiogram. M (male) and F (female) designations refer to G0 lines with selected G1 transformant progeny of samples.

Figure 3 (c) is an autoradiogram of a Southern DNA hybridization analysis of *Dm*[pB*w*, *gfp*] transformant sublines, and wild type (wt) and *w*[m] host strain control samples using *Xho*I digestion and *Hpa/Ase piggyBac* fragment as probe. DNA size markers are shown to the left of the autoradiogram. M and F designations refer to G0 lines with selected G1 transformant progeny of samples.

Figure 3 (d) is an autoradiogram of a Southern DNA hybridization analysis of *Dm*[pB*w*, *gfp*] transformant sublines, and wild type (wt) and *w*[m] host strain control samples using *Pst*I digestion and *Hpa/Ase piggBac* fragment + EGFP DNA as probe. DNA size markers are shown to the left of the autoradiogram. M and F designations refer to G0 lines with specific G1 line numbers are given below, with the designation (+) for those expressing visible eye pigmentation and (-) for those having non-pigmented white eyes.

Figures 4 (a) and (b) show inverse PCR strategy to isolate the pB[Dmw] vector insertion site in transformant sublines. Figure 4 (a) is a schematic (not to scale) of the vector insertion in the host plasmid showing the approximate location of the restriction sites and primers used for PCR. Forward (F) and reverse (R) primers are numbered according to their nucleotide position in *piggyBac*. The *piggyBac* sequence is shown in gray surrounded by the TTAA (SEQ ID NO 1) duplicated insertion site, the mini-*white* marker gene is white, and chromosomal sequence is hatched.

Figure 4(b) shows the *piggyBac* insertion site sequence in p3E1.2 (SEQ ID NOs 7 and 8), and the proximal insertion site sequences (SEQ ID NOs 9 and 10, 11 and 12, and 13 and 14) for three of the transformant sublines.

Figure 5 shows a circular map of the vector pB[PUb-nls-EGFP] #257.

Figures 6a-6f show SEQ ID NO 6 for pB[PUb-nls-EGFP]#257.

Figure 7(a) is a photomicrograph showing GFP expression in *Anastrepha suspensa* transformed with *piggyBac*/PUb-nls-EGFP at embryo stages. Under UV light, transformants exhibit bright green

fluorescence, with wild-type non-transformants exhibiting muted yellow autofluourescence (digital images taken with Leica MZ-12 fluorescence microscope and SPOT-1 CCD camera).

Figures 7(b) and 7(c) are photomicrographs showing GFP expression in *Anastrepha suspensa* transformed with *piggyBac*/PUb-nls-EGFP at larval stages. 7(b) is a wild-type non-transformant and 7c is a transformant. Under UV light, transformants exhibit bright green fluorescence, with wild-type non-transformants exhibiting muted yellow autofluourescence (digital images taken with Leica MZ-12 fluorescence microscope and SPOT-1 CCD camera).

Figures 7(d) and 7(e) are photomicrographs showing GFP expression in *Anastrepha suspensa* transformed with *piggyBac*/PUb-nls-EGFP at adult stages. 7(d) is a wild-type non-transformant and 7(e) is a transformant. Under UV light, transformants exhibit bright green fluorescence, with wild-type non-transformants exhibiting muted yellow autofluourescence (digital images taken with Leica MZ-12 fluorescence microscope and SPOT-1 CCD camera).

Figures 8(a)-8(e) are eye color phenotypes of *Bactrocera dorsalis* wild-type (+) and white eye (WE) host strain and the Bd[pBCcw] transformant lines 61,115, and 137.

Figures 9(a) and 9(b) show medfly, *Ceratitus capitata* transformed with *piggyBac*/white/EGFP vector (pB[Ccw,pUB-nls-EGFP]) expressing eye color under brightfield (9a) and GFP expression under ultraviolet (9b).

Figure 10 shows a transgenic insect having three integrations observed under ultraviolet light after various times after decaptitation. Flies were decapitated at day 0, taped in a plastic box placed outdoors in partial sunlight. Digital photographs were taken each day at the same exposure and magnification.

Detailed Description of the Invention

The present invention is an effective transformation system for producing transgenic organisms, especially transgenic insects. The identification and isolation of an autonomous *piggyBac* transposon enables transformation of cells and the production of transgenic organisms wherein DNA capable of being expressed in the

transformed cell or transgenic organism is excised from a transformation construct and inserted into the genome of a cell used to produce a transgenic organism (United States Patent Application 08/844,274; herein incorporated by reference). The term cell for the purposes of this invention includes any cell capable of being transformed by the transformation construct of the present invention and preferably includes any eukaryotic cell. The term organism for the purposes of the present invention includes any unicellular or multicellular living entity capable of being transformed by the transformation construct of the present invention and preferably includes multicellular eukaryotes. More preferably, the cell or organism is an insect cell or an insect.

The present invention utilizes the transposon machinery of the TTAA (SEQ ID NO 1) specific transposons to excise and insert a targeted functional heterologous DNA sequence into the genome of the host cell. The resulting transformed cell or group of cells are stable transformants that are then used to make a transgenic organism, using techniques known to the skilled artisan, that will pass the introduced gene to all subsequent progeny. The targeted functional heterologous DNA for purposes of this invention is any heterologous DNA capable of being expressed in a host cell and/or a transgenic organism.

The transformation system of the present invention includes a vector, such as, for example, pB[PUb-nls-EGFP] (Figures 5 and 6), that includes a modified *piggyBac* transposon (pB), a marker construct that includes the enhanced green fluorescent protein gene (EGFP) linked to the promoter region of the *Drosophila melanogaster* polyubiquitin (PUb) gene and the nuclear localizing sequence (nls) of the SV40 virus. This vector can be used to transform and detect transgenic organisms based on expression of the green fluorescent protein marker under ultraviolet light. After chromosomal integration and inheritance of the vector, expression of green fluorescent protein occurs in all cell types, is intense, strongly localized to nuclei, and continues to be detectable under ultraviolet light even after death of the organism. The novel features of this vector includes its construction that deletes about 748 bp of internal *piggyBac*

sequence without diminishing its function, and the function of the polyubiquitin promoter in a nondrosophilid species. This has utility as a broadly based method for the creation and selection of transgenic organisms, and as a genetic marker for detecting and tracking transgenic insects used in field release programs (Figure 5).

GFP expressivity is critical for nondrosophilid species not amenable to mutant-rescue, it also widens the possibility for using the dominant expression of GFP as a primary transformant marker in many *Drosophila* lines not already carrying the *white* or *rosy* mutations, or for screens requiring selection in early development. Though vectors carrying *white* and *gfp* have been tested previously, the transformations used only white as the transformant selection, with GFP assessed secondarily for specific spatial or developmental expression (Davis et al., Devel. Biol., Volume 170, 726-729, 1995; Wang & Hazelrigg, Nature, Volume 369,400-403, 1994).

The transformation system of the present invention also includes a *piggyBac* transposase helper plasmid, pBΔSac, having its' 5' terminus deleted as described by Handler et al. (1998, *supra*; herein incorporated by reference). A new transposase helper under heat-shock promoter regulation was created by the isolation of the 457 bp *Xba*I-*Xmn*I 5' nontranslated sequence from the *hsp*70 gene (Lis et al., Cell, Volume 35, 403-410, 1983, herein incorporated by reference). The heat-shock regulated helper increases the transformation frequency by eight-fold in *Drosophila*, indicating that the *piggyBac* system could be as effective as routinely used systems such as *P* and *hobo* that have been thus far inactive in nondrosophilids (O'Brochta & Atkinson, Insect Biochem. Molec. Biol., Volume 26, 739-753, 1996).

The creation of a transformed cell requires that the vector containing the functional heterologous DNA first be physically placed within the host cell. Current transformation procedures utilize a variety of techniques to introduce DNA into a cell. In one form of transformation for vertebrate systems, the DNA is microinjected directly into embryos through the use of

micropipettes. Alternatively, high velocity biolistics can be used to propel small DNA associated particles into the cell. In another form, the cell is permeablized by the presence of polyethylene glycol, thus allowing DNA to enter the cell through diffusion. DNA can also be introduced into a cell by fusing protoplasts with other entities that contain DNA. These entities include minicells, cells, lysosomes, or other fusible lipid-surfaced bodies. Electroporation is also an accepted method for introducing DNA into a cell. In this technique, cells are subject to electrical impulses of high field strength that reversibly permeabilizes biomembranes, allowing the entry of exogenous DNA sequences. One preferred method of introducing the transformation system of the present invention into insect embryos, in accordance with the present invention, is to microinject fertilized eggs with the vectors of the present invention. The DNA sequence flanked by the transposon inverted repeats will be inserted into the genome of some of the germ cells of the fertilized egg during development of the organism. This DNA will then be passed on to all of the progeny cells to produce transgenic organisms. The microinjection of eggs to produce transgenic animals has been previously described and utilized to produce transformed mammals and insects (Rubin et al., Science, Volume 218, 384-393, 1982; Hogan et al., Manipulating the Mouse Embryo: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Plainview, N.Y., 1986; Morgan et al., Annu. Rev. Biochem., Volume 62, 191-217, 1993; Spradling, A.C., In: Drosophila: A Practical Approach, ed. D.B. Roberts, Oxford: IRL Press, 175-197, 1986; all herein incorporated by reference). Accordingly, a method of producing stably transformed insects includes the step of microinjecting the transformation constructs of the present invention comprising the inverted repeats of a TTAA specific transposon and a helper construct into a cell, preferably a fertile insect egg. This is followed by incubation in an oxygenated and humidified tissue culture chamber at about 22-23° C for about 3-6 hours. Injected cells or eggs are then heat shocked at about 37°-41° C, about 39°C preferred, for about 1 hour. The resulting transformed cells or transgenic organisms have exogenous DNA inserted into the genomic DNA at the sequence TTAA.

Transformed cells and/or transgenic organisms can be selected

from untransformed cells and/or non-transgenic organisms by ultraviolet light since the transformation system includes an enhanced green fluorescent protein gene that produces an altered visible phenotype under ultraviolet light. Using standard techniques known to those familiar with the field, techniques such as, for example, Southern blotting and polymerase chain reaction, DNA can be isolated from transformed cells and/or transgenic insects to confirm that the introduced DNA has been inserted.

Genetic modification of insects with new genetic elements provides a means to control populations of agriculturally pestiferous or beneficial insects. The ability to control pest insects through genetically based sterile insect programs or genetically introduced targeted conditional susceptibilities will result in significant cost savings to agribusiness. This technology can also be used for detection and monitoring of insect populations and infestations where *piggyBac* transgenic insects are present in the population. In addition, introduction of genes that impart resistance to chemicals (including herbicides, pesticides, and insecticides) can improve the efficacy of beneficial insects. Each of these applications will result in more efficient pest control programs.

Enhancing the resistance of beneficial insects to pesticides will enhance the efficacy of the beneficial insects and may allow for the simultaneous use of chemical control and biological control of pests. Some of the beneficial insects that would make good candidates for such transformations include Hymenopteran parasitoids of Heliothis spp.: *Micropilitis croceips* and *Cardiochiles nigriceps*; Hymenopteran parasitoid of Diamondback moth, *Plutella xylostella*: *Diadegma insolare*; Hymenopteran parasitod of the Indianmeal moth, *Plodia interpunctella*: *Bracon hebitor*; and Hemipteran predators: *Xylocoris flavipes*, *Podisus maculatus*.

The following examples are intended only to further illustrate the invention and are not intended to limit the scope of the invention as described by the claims. *Drosophila melanogaster* white strain w[m], was used in the following examples

as a model system for transformation system studies using the vectors of the present invention. *D. melanogaster* and transformant progeny were maintained at about 23-25° C on standard cornmeal-yeast-molasses media.

EXAMPLE 1

The *piggyBac* transposase helper plasmid, pBΔSac, having its 5' terminus deleted was described previously (Handler et al., 1998; *supra*, herein incorporated by reference). pBΔSac was created by digestion of p3E1.2 (United States Patent Application 08/844,274) with *Sac*I and religation, that deletes the 5' *piggyBac* terminal sequences but maintains the putative *piggyBac* promoter region. A transposase helper under heat-shock promoter regulation was created by isolation of the 457 bp *Xba*I-*Xmn*I 5' nontranslated sequence from the *hsp*70 gene (Lis et al., 1983, *supra*; herein incorporated by reference). The *Xba*I-*Xmn*I fragment was blunted and ligated into the *Sac*I-blunted site of pBΔSac to create phsp-pBac. This places the *hsp*70 promoter sequence upstream of the putative *piggyBac* promoter.

The pB[Dmw] vector was created by insertion of a *Drosophila melanogaster* mini-*white* gene (Pirrotta et al., EMBO J., Volume 4, 3501-3508, 1985; herein incorporated by reference) into the 3E1 *piggyBac* element within the 6.0 kb p3E1.2 plasmid (Cary et al., 1989, *supra*). The mini-*white* gene was isolated as a 4.2 kb *Eco*RI fragment, blunted and ligated into the p3E1.2 *Hpa*I site. The inserted *w* gene interrupts the *piggyBac* open reading frame (ORF), but otherwise leaves the *piggyBac* element intact, with the respective promoters in opposite orientation. A *piggyBac* vector marked with *w* and *gfp* was created by initial construction of *piggyBac* marked with an enhanced *gfp* regulated by *D. melanogaster* polyubiquitin (PUb) promoter (Lee et al., Mol. Cell. Biol., Volume 8, 4727-4735, 1988; herein incorporated by reference) linked in-frame to the SV40 nuclear localizing sequence (nls) (Lanford et al., Mol. Cell. Biol., Volume 8, 2722-2729, 1986). The

polyubiquitin-nls (PUb-nls) cassette from PUbnlsGFP (Davis et al., 1995, *supra*) was isolated as *Kpn*I-*Sma*I fragment and inserted into the *Kpn*I-*Sma*I cloning site of EGFP-1 (Clontech) (Cormack et al., Gene, Volume 173, 33-38, 1996; Yang et al., Nucleic Acid Res., Volume 24, 4592-4593, 1996). Polyubiquitin-nls-EGFP was then isolated as a 4.1 kb *Bgl*II-*Stu*I fragment and ligated into the *Bgl*II-*Hpa*I site of *piggyBac* within p3E1.2 to create pB[PUbnlsEGFP]. The *Bgl*II-*Hpa*I digestion results in a 748 bp deletion within p3E1.2. The mini-*white* gene was then inserted into the unique *Bgl*II site by blunt-end cloning to create pB[Dmw, PUbnlsEGFP].

EXAMPLE 2

Embryo injections used standard procedures (Rubin & Spradling, Science, Volume 218, 348-353, 1982; herein incorporated by reference) with dechorionation achieved either manually or by 1.6% hypochlorite solution followed by about 2 washings in approximately 0.02% Triton-X 100 in water. Eggs were placed on double-stick tape, desiccated in room-air for about 10-15 minutes and submerged under Halocarbon 700 oil. Injections followed standard *Drosophila* microinjection procedures (Rubin and Spradling, Science, Volume 218, 348-353, 1982; herein incorporated by reference). DNA mixtures had vector:helper concentrations of about 600:400 µg/ml, respectively, in injection buffer (approximately 5 mM KCl; approximately 0.1mM sodium phosphate; at about pH 6.8). Injected eggs were placed in an oxygenated and humidified tissue culture chamber at about 22-23°C for about 3-6 hours, and phsp-pBac injected eggs were heat shocked at about 37°C for about one hour. Hatched larvae were collected about 1-2 days later and placed on larval diet. Eclosed G0 male adults were mated either individually to about 2 or 3 w[m] virgin female adults, or in groups of about three females to about six males. G1 eggs were collected for two weeks and reared under standard conditions that include maintaining the eggs at about 23-25° C on standard cornmeal-yeast-molasses media (Ashburner et al., *supra*).

Green fluorescent protein (GFP) was observed at all

developmental stages under a Leica MZ-12 stereozoom microscope using a mercury lamp and a ultraviolet longpass filter set (HQ 41012 FITC; Chroma) optimized for red-shifted GFP variants. Photographic documentation used an Olympus OM-4 camera and 400 ASA Fujichrome film with exposure times that were determined empirically.

In the first of three transformation experiments, the *piggyBac* vector system was tested in *D. melanogaster* white strain using a helper transposase under *piggyBac* regulation (pBΔSac) and a vector marked solely with *D. melanogaster* mini-*white* gene (pB[Dmw]). A mixture of vector and helper plasmids at concentrations of about 600 and about 400 µg/ml, respectively, was injected into about 2,650 embryos from that about 418 larvae hatched with about 283 emerging as adults. (See Table 1 below). The G0 adults were backcrossed to *w*[m] flies in groups totaling about 111. Four of the G0 lines yielded G1 offspring having varying levels of eye pigmentation (Figure 1). One line (F30) was sterile, and one line produced only white eye offspring, and therefore only two of the putative Dm[pBw] transformants were verified. One of these (F13) exhibited eye pigmentation only in females in several succeeding generations, suggesting that the integration caused a sex-linked lethal mutation. Presuming a fertility rate of about 50% (fertility rates are typically between about 40-60%; see below), an approximate transformation frequency of about 1-3% of fertile G0s was obtained.

In a second experiment, the pB[Dmw] vector was again tested but with a *piggyBac* transposase helper under *D. melanogaster hsp*70 (Lis et al., 1983 *supra*) promoter regulation (phsp-pBac). A vector/helper mixture, at a concentration of approximately 600/400 µg/ml was injected into about 1,940 embryos, of which about 247 larvae hatched, with about 122 emerging as adults (See Table 1, below). G0 adults were initially backcrossed in a total of about 49 groups to *w*[m] flies, after which they were individually mated to determine fertility. Of the about 98 surviving G0 flies, about 41 yielded offspring resulting in a fertility rate of about 42%. Of the 41 fertile G0 flies, 11 lines produced offspring having

varying levels of eye coloration (Figure 1) yielding a transformation frequency of about 26%. The number of G1 offspring from the G0 lines varied considerably, ranging from 1 G1 in lines M11 and F1, to 102 G1 flies in line M13.

In a third experiment, the phsp-pBac helper was used, but with a *piggyBac* vector including the enhanced green fluorescent protein (gfp) marker gene in addition to the *D. melanogaster* white gene. This allowed the testing of a new gfp marker construct in transformants that could be primarily identified by white expression. Although expression of wild type GFP under polyubiquitin-nuclear localizing sequence regulation had been tested previously in *D. melanogaster P* transformants (Davis et al., 1995, *supra*), the vector of the present invention improves expression of GFP by using an enhanced GFP (EGFP-1) having a double mutation causing a reported increase in expression of up to about 35-fold (Cormack et al., 1996, *supra*; Yang et al., 1996, *supra*). The variant form is also optimized for mammalian codon usage and polyadenylation, and preliminary tests of the marker construct indicated transient GFP expression in both *Drosophila* embryos and dipteran and lepidopteran cell lines (A.M. Handler and R.A. Harrell, unpublished). The vector construct, pB[Dmw, PUbnlsEGFP], also allowed evaluation of *piggyBac* transformation with about a 10.0 kb vector, approximately 3.4 kb larger than previous vectors tested, and having about 748 bp of *piggyBac* DNA deleted (previous vectors retained all *piggyBac* DNA). As before, a mixture of about 600 µg/ml vector and about 400 µg/ml helper was injected into about 2147 embryos, of which about 412 larvae hatched, and about 218 emerged as adults (Table 1 below). G0 adults were backcrossed to *w*[m] flies in a total of about 90 mating groups, of which about 79 yielded offspring. Although *white*$^{+}$ gene expression (eye pigmentation) was depended upon as the primary marker, G1 larvae and pupae were examined under UV for visible GFP expression, and seven of the G0 lines yielded fluorescent G1 larvae and pupae. Interestingly, as shown below in Table 2, upon adult emergence only six of the seven G0 lines

yielded G1 offspring with observable eye color pigmentation. While about 70 G1 offspring in total exhibited observable green fluorescence, only about 27 of these flies exhibited a level of eye pigmentation that would have allowed their selection under normal screening procedures. In contrast, all of the G1 flies with eye color pigmentation expressed GFP. Figure 1b shows a Dm[pBw, *egfp*] transformant having an orange eye color and GFP fluorescence, with no fluorescence observed in the w[m] host. Figure 1c shows another transformant having a white eye phenotype indistinguishable from that in the w[m] host strain, but exhibiting an equal, if not greater level of GFP fluorescence compared to the orange eye transformant. Notably, fluorescence is quenched in the eye of the pigmented transformant, while it is easily visible in the white eye transformant. High magnification examination revealed a few pigmented ommatidia in some white eye G1 flies expressing GFP, though these would not have been normally detected. Based on selection by GFP expression and presuming about 50% fertility, an approximate transformation frequency of about 6-7% of fertile G0 flies is deduced.

An assessment of vector activity based on germline transformation frequency is a factor of both transposon mobility in the host embryo and levels of genomic position effect suppression of the marker gene, or stated more simply, the ability to visibly identify putative transformants. While position effect variegation and suppression of *white* expression in transformants is well established (Hazelrigg et al., Cell, Volume 64, 1083-1092, 1984; Pirotta et al., 1985, *supra*), the effect of complete marker suppression on transformation frequencies has not been assessed since such transformants have been only detected fortuitously after molecular analysis. The experiment using both the *white* and GFP markers proved the importance of position effects on marker expression convincingly, since GFP was readily detectable in 70 G1 flies, yet eye pigmentation was apparent in less than 40% of these. Under typical screening procedures these flies would not have been scored as transformants, though pigmentation in a few ommatidia in some flies could be detected at high magnification, and for a few lines, pigmentation was more apparent in subsequent

generations. It is likely that expression of the *white* marker would have been improved by heat shock regulation, but nonetheless, GFP was easily detected in all the non-pigmented transformants, and strongly expressed in some. The influence of modifier genes on position effect variegation is complex, and target genes (or their promoters) are not equivalently affected (Bhadra et al., Genetics, Volume 150, 251-263, 1998). The polyubiquitin-*gfp* gene may be a target of position effect modifiers, but it is clearly less susceptible to suppression relative to *white* in terms of its expressed phenotype in the same chromosomal context. The data suggests that GFP is a more reliable visible marker than *white*, that portends well for its use as a general marker in other insects.

TABLE 1. Transformation Experiments.

Expt	vector/ helper	eggs injected	G0s mated	% fertility	No. G0 lines	No. G1 lines	transformant frequency
I	pB[Dm*w*]/pBΔSac	2,650	283	nd	4	11	0.01-0.03*
II	pB[Dm*w*]/phsp-pBac	1,940	122	42	11	266	0.26
III	pB[Dm*w*, PUbnlsEGFP]/phsp-pBac	2,147	218	nd	7	70	0.06-0.07*

*estimated frequency based on 50% fertility

TABLE 2. G1 *white*$^+$ and GPR marker expression in Dm[pBw, gfp] transformants.

G0 line	No. G1	GFP	white$^+$	Frequency *white*$^+$
M4	4	4	3	0.75
M9	21	21	2	0.10
M12	3	3	1	0.33
M23	15	15	14	0.93
M45	5	5	0	0
M47	21	21	6	0.29
F10	1	1	1	1.00
Total	70	70	27	0.39

EXAMPLE III

Southern hybridization was performed to verify genomic transposition of the *piggyBac* vectors. Approximately 5-10 µg of genomic DNA was digested with indicated restriction enzymes and separated on about 0.8% agarose gels. DNA was stained with ethidium bromide, blotted to nylon filters and immobilized by ultraviolet irradiation. Hybridization probes were labeled with [^{32}P]-dCTP by random priming (Gibco BRL) according to the manufacturer's specifications. Probe DNA was generated from indicated *piggyBac* restriction fragments (see below) that were separated from p3E1.2, or the entire egfp gene from pEGFP-1 (Clontech) by agarose electrophoresis and gel-elution. Hybridizations were performed in phosphate buffer, approximately pH 7.5; about 1% BSA; about 7% SDS at about 65°C with an initial wash in about 2X SSC; about 0.2% SDS at about room temperature and about two washes in about 1X SSC; about 0.1% SDS at about 55°C for approximately 30 minutes. Autoradiography was performed by exposure of Kodak X-Omat film at about -90° C.

Genomic transposition of the *piggyBac* vectors was verified by Southern DNA hybridization. The basic strategy was to perform hybridizations to the 5' vector arm using the *piggyBac* *Sph*I-*Hpa*I or *Nsi*I-*Hpa*I fragment as probe, and the 3' vector arm using the *Hpa*I-*Ase*I or *Hpa*I-*Nsi*I fragment as probe. Using probes to both

vector arms, internal fragments spanning most of the vector were detected. Hybridizations to the vector arms and adjacent chromosomal sequence indicate their presence in non-plasmid DNA and indicate the number of integrations, while internal hybridizations that yield known fragment sizes confirm vector integrity.

For pB[Dmw] transformants, genomic DNA was initially digested with *Bgl*II and hybridized to the labeled *Sph-Hpa piggyBac* fragment, that detects both vector arms resulting in two bands for each integration (Figure 2A). Each intact vector integration should result in one band greater than about 0.67 kb for the 5' arm, and one band greater than about 5.9 kb for the 3' arm. Since varying eye color phenotypes among G1 sublines was observed, and in some cases within G1 sublines, sublines having light orange, dark orange, or red eye coloration from the same G1 sublines were selected for hybridization analysis. For example, flies having differing phenotypes from lines M13-39, M19-90, and M19-91 were hybridized separately, but no difference in the number or sites of insertion were apparent. Of all the lines tested, all had single integrations except for two lines having two integrations (M13-39 and M19-91) and one line having three integrations (F14-63). All the lines with multiple integrations had dark orange or red eye color, though several lines with a single integration also shared these phenotypes. Hybridization patterns for the lines tested indicated that for most of the G0 lines, different integrations were transmitted to many of the G1 sibling offspring. For example, the three G1 sublines tested from both the M3 and M5 G0 lines all show different patterns indicating at least three independent integrations occurring in the two G0 germlines.

Genomic DNA digested with *Sal*I and hybridized to *Hpa*I-*Ase*I probe yielded single bands greater than about 3.0 kb for each integration, and the number of integrations determined were consistent with the *Sph*I-*Hpa*I hybridizations (Figure 2B). For all samples, *Nsi*I digestion and hybridization to *Nsi-Hpa*I and *Hpa*I-*Nsi*I probe yielded only about 1.5 kb and about 4.6 kb bands accounting for about 6.1 kb of the about 6.6 kb vector, indicating the same generally high level of vector integrity for all

integrations tested.

G1 sublines from six G0 lines transformed with the pB[Dmw, PUbnlsEGFP] vector were digested with either *Bgl*II and probed with *Sph*I-*Hpa*I *piggyBac* DNA for 5' vector arm analysis, or digested with *Xho*I and probed with *Hpa*I-*Ase*I *piggyBac* DNA for 3' arm analysis (Figure 3A and 3B). Both hybridizations yielded one band for each sample, indicating single integrations having occurred in each line. *Nsi*I restriction digests with *Nsi*I-*Hpa*I and *Hpa*I-*Nsi*I hybridizations yielded about 0.7 kb and about 0.8 kb bands indicating vector integrity for each integration (data not shown).

Two G0 lines, M9 and M47, yielded a high proportion of G1 flies expressing only GFP and white eyes, and line M45 that yielded only white eye transformants. These lines were analyzed by *Pst*I digestion and hybridization to EGFP and *Hpa-Ase*. All lines shared the about 4.4 kb internal vector fragment, with an additional junction fragment from the 3' vector arm and adjacent insertion site chromosomal DNA. The M9 white eye lines all shared the same integration indicated by an about 0.9 kb junction fragment, and similarly the M47 white eye lines all shared the same 5.0 kb junction fragment. The pigmented lines M9-2 and M9-3 had different integrations from each other, and from their white eye sibling lines, and the pigmented lines M47-9 and M47-10 shared the same integration based on an about 4.0 kb junction fragment, but which differs from their white eye siblings. These hybridizations, and that for M45-1, proves that the white eye flies were transformed, and that white expression was likely influenced by differing insertion sites from their pigmented sibling lines.

EXAMPLE IV

To verify that *piggyBac*-mediated chromosomal transpositions had occurred, insertion sites were isolated by inverse PCR from sublines F1-2, M17-4 and M31-6, all having single integrations. Inverse PCR was performed as described previously (Handler et al., 1998, *supra*; herein incorporated by reference) using *Hae*III digestions for 5' and 3' junctions and *Msp*I digestion for 3'

junctions. After about 4 hours digestion, restriction fragments were circularized by ligation at about 16° C for about 16 hours. PCR was preformed on the circularized fragments by using primer sequences in opposite orientation within the *piggyBac* restriction site and terminus for each junction. For the 5' junction, the forward primer (572F) 5'-TCTTGACCTTGCCACAGAGG-3' (SEQ ID NO 2) and reverse primer (154R) 5'-TGACACTTACCGCATTGACA-3' (SEQ ID NO 3) were used. For the 3' junction the reverse primer (2118R) 5'-GTCAGTCCAGAAACAACTTTGGC-3' (SEQ ID NO 4) and the forward primer (2385F) 5'-CCTCGATATACAGACCGATAAAAACACATG-3' (SEQ ID NO 5) were used. PCR products were separated in low-melting-temperature agarose, and fragments were selected that were longer than the respective restriction site terminus distances and different from those expected from the p3E1.2 based vector and helper plasmids. These products were directly subcloned into ddT vectors (Invitrogen), that were sequenced by using primers to vector sequence proximal to the respective termini. Subcloned PCR products were sequenced and analyzed by alignment using GeneWorks 2.5 software (Oxford Molecular Group) and subjected to BLAST analysis (Altshul et al., J. Mol. Biol., Volume 215, 403-410, 1990; herein incorporated by reference) to identify genomic insertion site sequences and distinguish them from those in the injected plasmids. For all the integrations both the 5' and 3' junctions yielded the *piggyBac* inverted terminal repeat sequences immediately adjacent to a TTAA sequence (SEQ ID NO 1) and proximal insertion site DNA (Figure 4). The TTAA (SEQ ID NO 1) duplicated target site is characteristic of all *piggyBac* integrations (Elick et al., Genetica, Volume 97, 127-139, 1995) and typically indicates a vector-mediated transposition. The BLAST analysis revealed that the M17-4 integration occurred in a TTAA site within the *cubitus interruptus*-Dominant gene located on chromosome 4 at nucleotide 12,898 (GenBank submission U66884; Ahmed & Podemski, Gene, Volume 197, 367-373, 1997), and the M3106 integration was found to have occurred in a TTAA site within a previously sequenced region of the distal X chromosome (GenBank submission AL09193; Murphy et al, direct submission). Determination of insertions in these previously sequenced sites gives the first

direct proof that a *piggyBac* vector does indeed insert into and duplicates TTAA (SEQ ID NO 1) insertion sites in a eukaryotic genome.

Two of the three insertion sites that were sequenced were found to be in previously sequenced genomic loci, and as expected, the insertions sites were all TTAA (SEQ ID NO 1) with one of them within the ci^D allele on the fourth chromosome. Many transposons have insertion site preferences, and for at least some, a clear negative bias against specific sites or loci. This has been clearly demonstrated by genomic hotspots and coldspots for *P* integration in *D. melanogaster* (See Engels, In: *Mobile DNA*, D.E. Berg and M.M. Howe, eds., American Society of Microbiology, Washington, D.C., 439-484, 1989), and by differences in preferential integration sites between *hobo* and *P* (Smith et al., Genetics, Volume 135, 1063-1076, 1993). If the TTAA (SEQ ID NO 1) specificity for *piggyBac* integration is not further influenced by proximal sequences, then *piggyBac* transpositions may find use in transposon-mutagenesis and enhancer traps for loci refractory ot *P* or *hobo* transpositions in *Drosophila*.

Example V

The Caribbean fruit fly, *Anastrepha suspensa*, was transformed with a *piggyBac* vector marked solely with PUb-nls-GFP(pB[PUb-nsl-EGFP]) (Figures 5 and 6) using the *hsp70-piggyBac* (phsp-pBac) helper. From injected embryos, 561 surviving G0 adults were intermated in 60 small groups. Four of the G0 groups yielded a total of 57 G1 offspring exhibiting green fluorescence at all stages of developement (See Figure 7) and chromosomal vector integrations were verified by Southern hybridization for each G0 group. To test GFP as a genetic marker for field released transgenic flies, the perdurance of GFP expression was assayed in transgenic flies killed by decapitation. Two to three day old *A. suspensa* adults transformed with pB[PUb-nls-EGFP], and wild type non-transformed adults, were decapitated and placed within a plastic box kept outdoors in partial shade. GFP fluorescence was

observed daily by digital images taken with a SPOT-1 cooled CCD digital camera (Diagnostic Instruments, Inc.) through a Leica MZ-12 stereozoom microscope. All images were taken at the same magnification and exposure parameters. Figure 10 shows that while GFP fluorescence decreases with time after death, unambiguous detection of GFP is still possible at 28 days after decapitation, with no fluorescence detectable in wild flies. This indicates that the PUb-nls-EGFP marker should be a reliable visible detection system for released transgenic insects, and especially for those captured and killed in field traps with monitoring occurring after extended time periods.

Example VI

A *piggyBac* vector marked with the Mediterranean fruit fly (*Ceratitis capitata*) white gene cDNA (pB[Ccw]) and the phsp-pBac helper was used to transform the oriental fruit fly (*Bactrocera dorsalis*). Injected G0 embryos from the *B. dorsalis* white eye mutant strain yielded 102 fertile adults, that upon individual backcrossing, yielded three lines of putative transformants with pigmented eyes (Figures 8a-8e). One of these lines produced 119 G1 transformants. Southern DNA hybridization analysis with *piggyBac* and *white* gene probe verified chromosomal integration of the *piggyBac-white* vector in all three lines. In a separate experiment, the *white*/PUb-nls-EGFP marker within pB[Ccw, PUb-nls-EGFP] was introduced into a single *B. dorsalis* transformant line from 17 G0 matings. As in Drosophila, the transformant was selected solely by GFP expression, having undetectable eye coloration. This reaffirms the notion that the polyubiquitin-EGFP marker is significantly more reliable than *white* gene markers.

Example VII

The PUb-nls-EGFP marker was introduced into the medfly, *Ceratitis capitata*, to further test GFP as a transgenic selection, and to create GFP-marked strains for testing as a field release marker in medfly SIT. First a *piggyBac* vector marked with PUb-

nls-GFP and the medfly *white* gene (pB[Ccw, PUb-nls-EGFP]) was tested, and then the vector solely marked with Pub-nls-GFP pB[PUb-nls-EGFP]) was tested. Both experiments used the *hsp70-piggyBac* (phsp-pBAC) helper. Based on GFP fluorescence, the first experiment yielded five transformant lines from 99 fertile G0s (See Figure 9), while the second experiment yielded three transformed lines from 17 fertile G0s. Transformation was verified by Southern hybridization analysis. The foregoing detailed description is for the purpose of illustration. Such detail is solely for that purpose and those skilled in the art can make variations without departing from the spirit and scope of the invention.

We claim:

1. A transformation system comprising a vector containing a nucleotide sequence from a piggyBac transposon in which is inserted an enhanced green fluorescent protein gene linked to a promoter region of a polyubiquitin gene and a nuclear localizing sequence of an SV40 virus.

2. A transformation system of claim 1 further comprising a *piggyBac* transposase helper plasmid under heat-shock promoter regulation.

3. The transformation system of claim 1 wherein said polyubiquitin gene is from *Drosophila melanogaster*.

4. The transformation system of claim 2 wherein said polyubiquitin gene is from *Drosophila melanogaster*.

5. The transformation system of claim 1 wherein said *piggyBac* transposon is modified by deleting about 748 bp of internal *piggyBac* sequence.

6. A vector having SEQ ID NO 6.

7. A transgenic organism transformed using the transformation system of claim 3 wherein in said organism is detectable under ultraviolet light.

8. A transgenic insect transformed using the transformation system of claim 3 wherein said insect is detectable under ultraviolet light.

9. The transgenic insect of claim 8 that continues to be detectable under ultraviolet light after death.

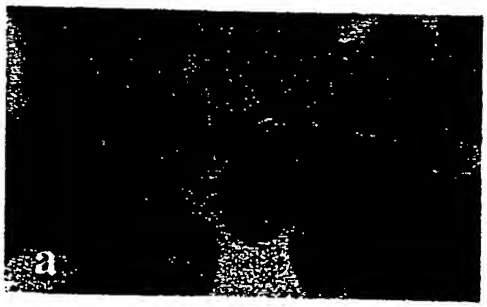

FIG. 1a

FIG. 1b

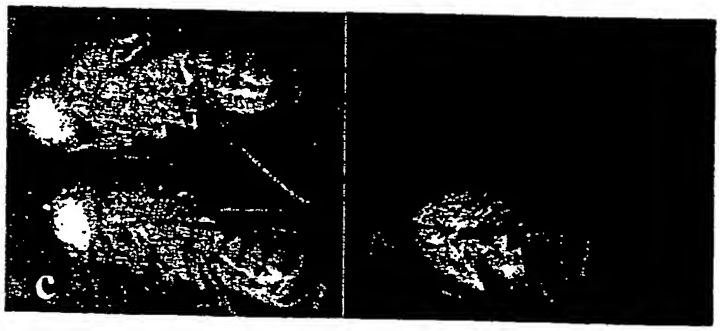

FIG. 1c

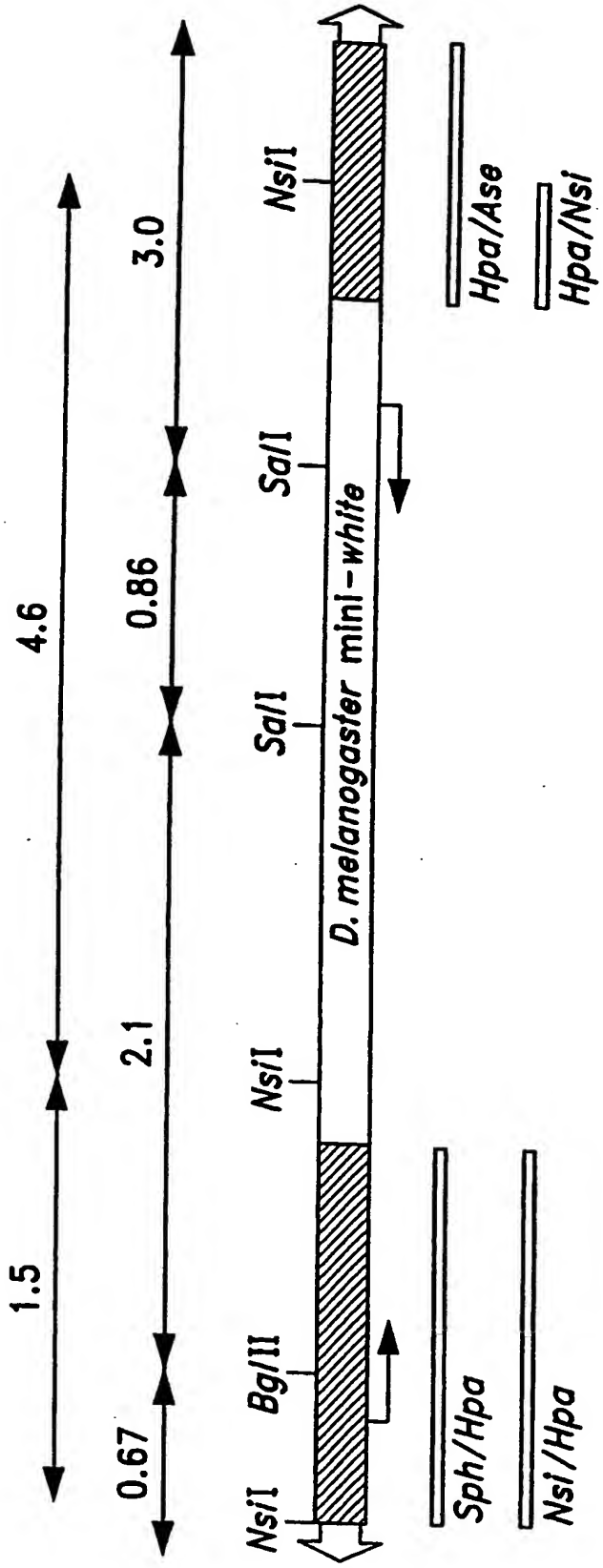
4.6
1.5
3.0
0.86
2.1
0.67
NsiI
SalI
SalI
NsiI
BglII
NsiI
D. melanogaster mini-white
Hpa/Ase
Hpa/Nsi
Sph/Hpa
Nsi/Hpa

Fig. 2a

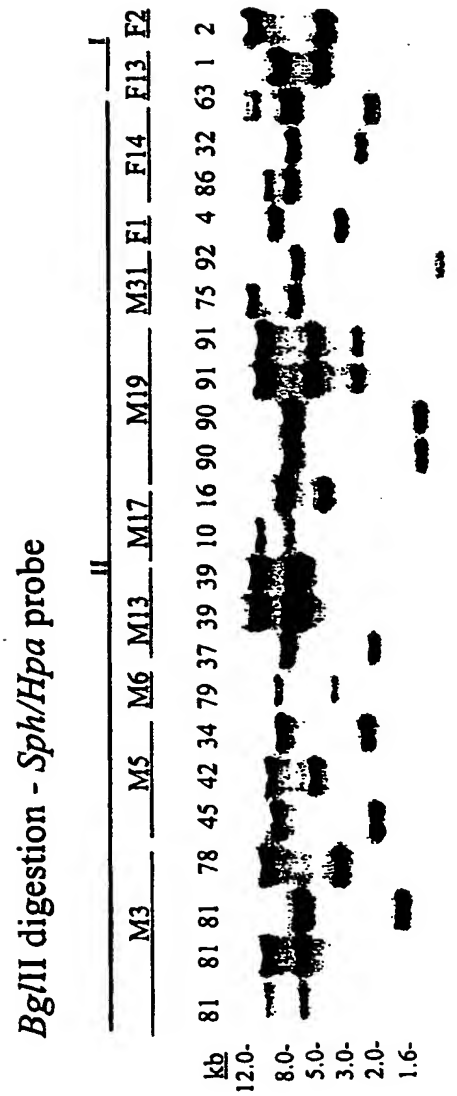
BglII digestion - Sph/Hpa probe
II
I
M3 M5 M6 M13 M17 M19 M31 F1 F14 F13 F2
kb 81 81 81 78 45 42 34 79 37 39 39 10 16 90 90 91 91 75 92 4 86 32 63 1 2
12.0-
8.0-
5.0-
3.0-
2.0-
1.6-

FIG. 2b

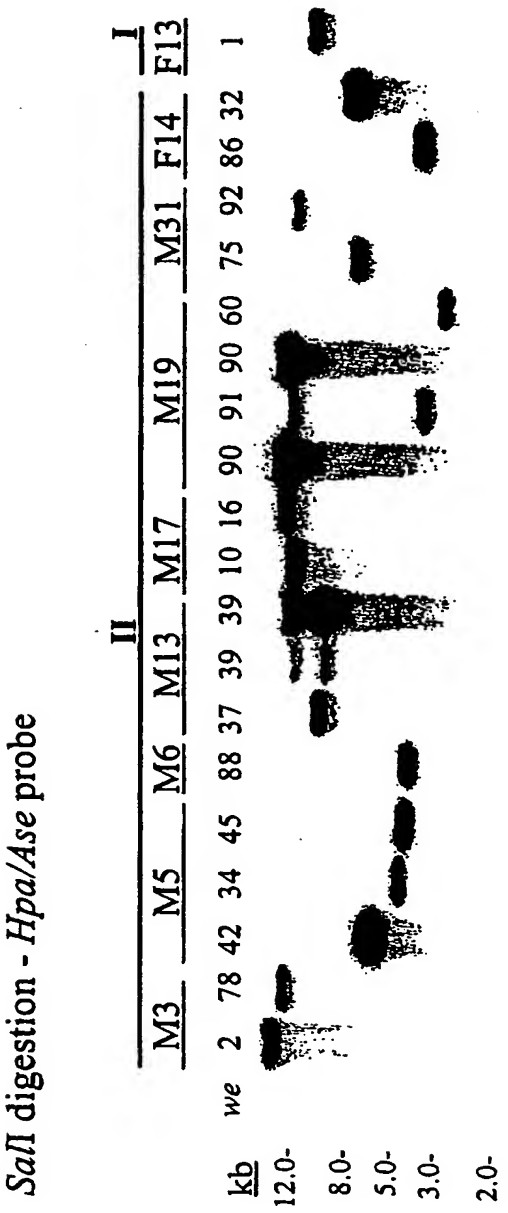
SalI digestion - Hpa/Ase probe
II
I
M3 M5 M6 M13 M17 M19 M31 F14 F13
kb we 2 78 42 34 45 88 37 39 39 10 16 90 91 90 60 75 92 86 32 1
12.0-
8.0-
5.0-
3.0-
2.0-

FIG. 2c

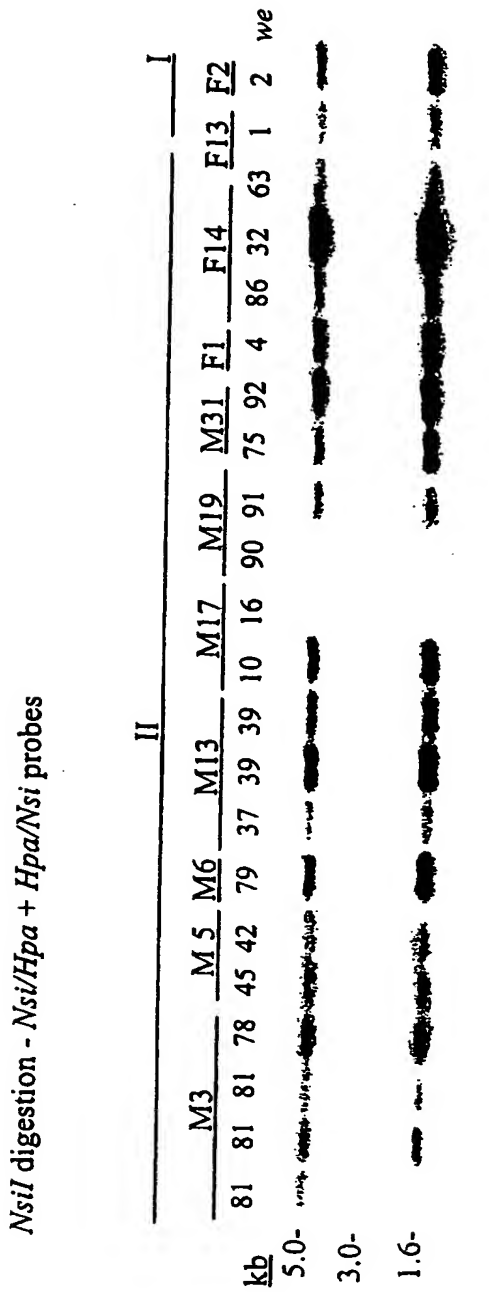
NsiI digestion - Nsi/Hpa + Hpa/Nsi probes
II
I
M3 M5 M6 M13 M17 M19 M31 F1 F14 F13 F2
81 81 78 45 42 79 37 39 39 10 16 90 91 75 92 4 86 32 63 1 2 we
kb
5.0-
3.0-
1.6-

FIG. 2d

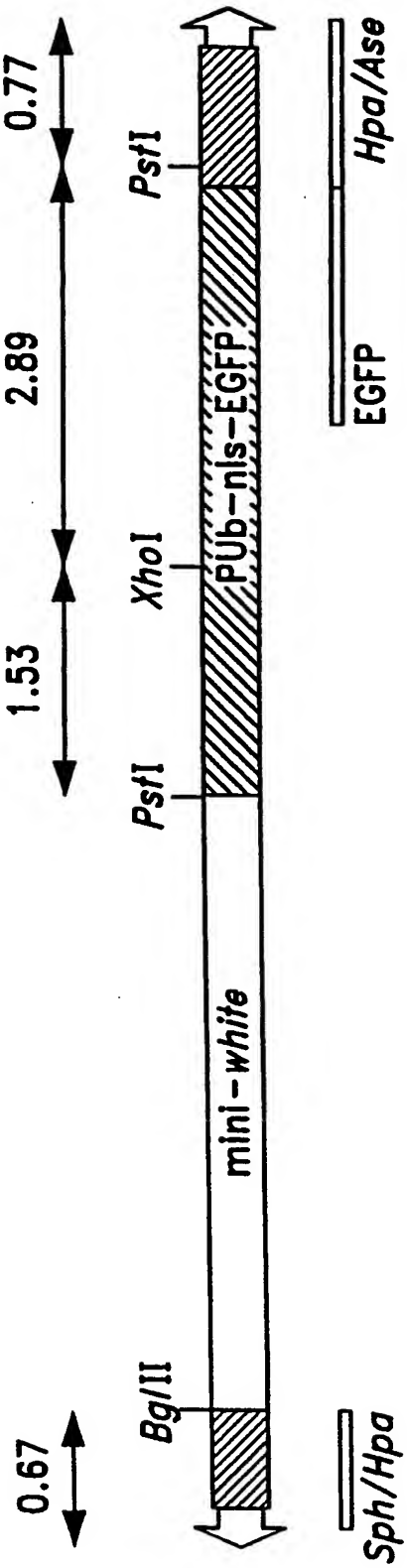
0.77
2.89
1.53
0.67
PstI
XhoI
PstI
Bgl II
PUb-nls-EGFP
mini-white
Hpa/Ase
EGFP
Sph/Hpa

Fig. 3a

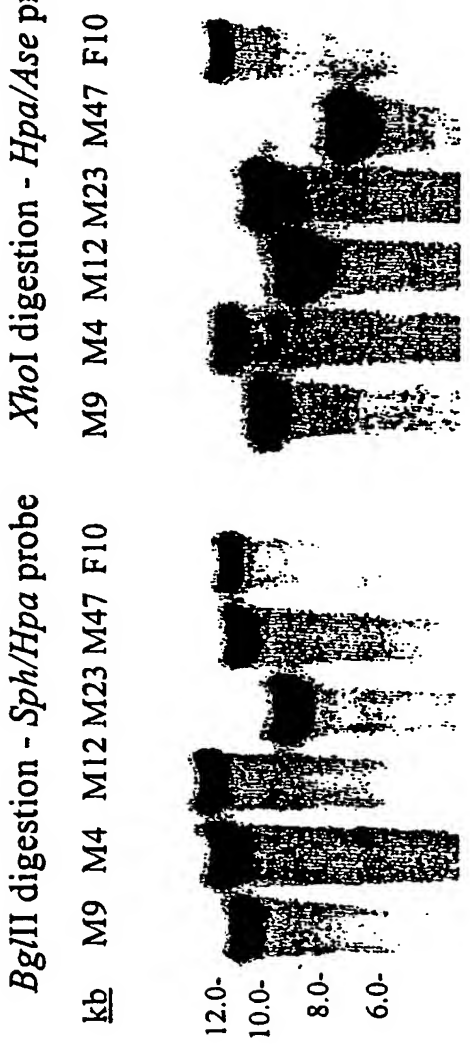
BglII digestion - Sph/Hpa probe
kb M9 M4 M12 M23 M47 F10
12.0-
10.0-
8.0-
6.0-
XhoI digestion - Hpa/Ase probe
M9 M4 M12 M23 M47 F10 we wt

FIG. 3b

FIG. 3c

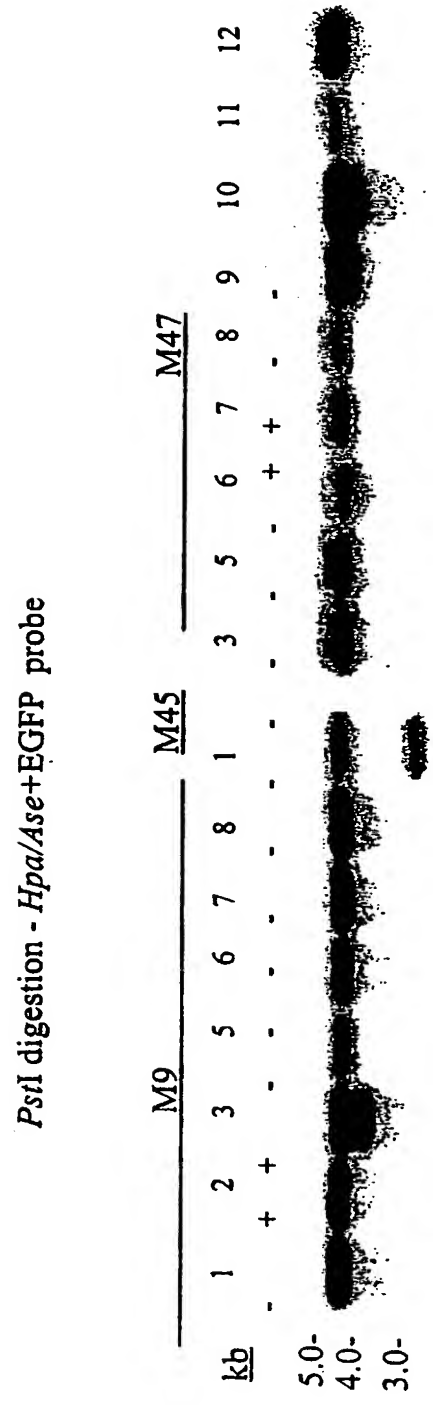
PstI digestion - Hpa/Ase+EGFP probe
M9
M45
M47
kb
1 2 3 5 6 7 8 1 3 5 6 7 8 9 10 11 12
- + + - - - - - - - - - + + - - -
5.0-
4.0-
3.0-
2.0-
1.0-

FIG. 3d

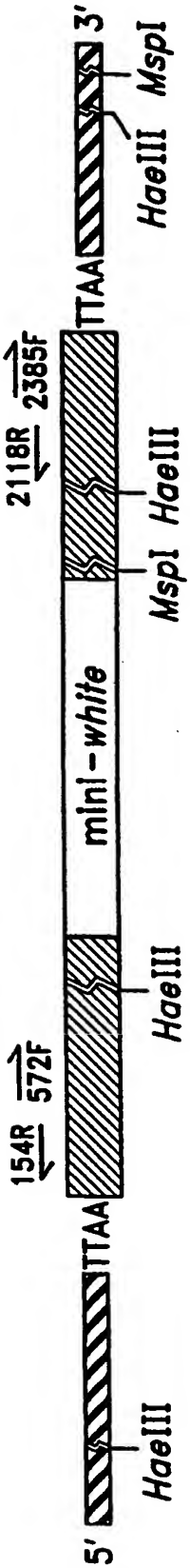
5'
HaeIII
TTAA
154R
572F
HaeIII
mini-white
MspI
HaeIII
2118R
2385F
TTAA
HaeIII
MspI
3'

Fig. 4a

p3E1.2 aagcgcaaatcttttttTTAA-*piggyBac*-TTAAataaatagtttctaat

F1-2 aaaaagactgactatTTAA-*piggyBac*-TTAAtaagcacactgagtc

M17-4 aaaatgtcgtctaggTTAA-*piggyBac*-TTAAagccgtatatcagat

M31-6 aaatgaacgacttttttTTAA-*piggyBac*-TTAAtggtttttagttgt

FIG. 4b

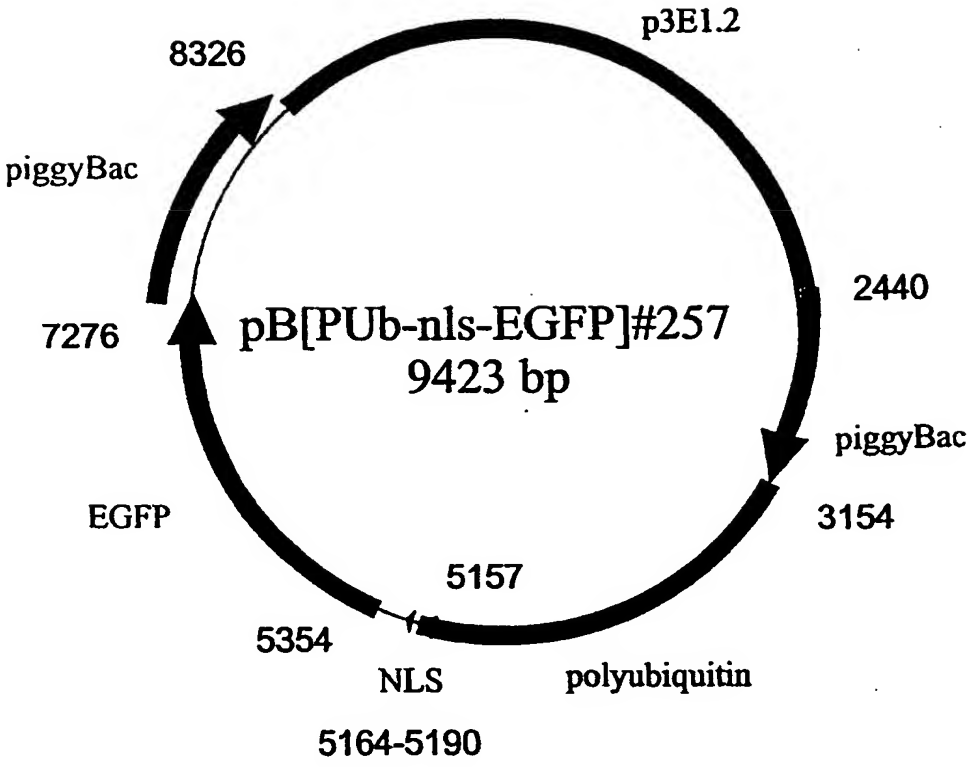
p3E1.2
8326
piggyBac
2440
pB[PUb-nls-EGFP]#257
9423 bp
7276
piggyBac
3154
EGFP
5157
5354
NLS
polyubiquitin
5164-5190

FIG. 5

10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
GACGAAAGGG	CCTCGTGATA	CGCCTATTTT	TATAGGTTAA	TGTCATGATA	50
ATAATGGTTT	CTTAGACGTC	AGGTGGCACT	TTTCGGGGAA	ATGTGCGCGG	100
AACCCCTATT	TGTTTATTTT	TCTAAATACA	TTCAAATATG	TATCCGCTCA	150
TGAGACAATA	ACCCTGATAA	ATGCTTCAAT	AATATTGAAA	AAGGAAGAGT	200
ATGAGTATTC	AACATTTCCG	TGTCGCCCTT	ATTCCCTTTT	TTGCGGCATT	250
TTGCCTTCCT	GTTTTTGCTC	ACCCAGAAAC	GCTGGTGAAA	GTAAAAGATG	300
CTGAAGATCA	GTTGGGTGCA	CGAGTGGGTT	ACATCGAACT	GGATCTCAAC	350
AGCGGTAAGA	TCCTTGAGAG	TTTTCGCCCC	GAAGAACGTT	TTCCAATGAT	400
GAGCACTTTT	AAAGTTCTGC	TATGTGGCGC	GGTATTATCC	CGTATTGACG	450
CCGGGCAAGA	GCAACTCGGT	CGCCGCATAC	ACTATTCTCA	GAATGACTTG	500
GTTGAGTACT	CACCAGTCAC	AGAAAAGCAT	CTTACGGATG	GCATGACAGT	550
AAGAGAATTA	TGCAGTGCTG	CCATAACCAT	GAGTGATAAC	ACTGCGGCCA	600
ACTTACTTCT	GACAACGATC	GGAGGACCGA	AGGAGCTAAC	CGCTTTTTTG	650
CACAACATGG	GGGATCATGT	AACTCGCCTT	GATCGTTGGG	AACCGGAGCT	700
GAATGAAGCC	ATACCAAACG	ACGAGCGTGA	CACCACGATG	CCTGTAGCAA	750
TGGCAACAAC	GTTGCGCAAA	CTATTAACTG	GCGAACTACT	TACTCTAGCT	800
TCCCGGCAAC	AATTAATAGA	CTGGATGGAG	GCGGATAAAG	TTGCAGGACC	850
ACTTCTGCGC	TCGGCCCTTC	CGGCTGGCTG	GTTTATTGCT	GATAAATCTG	900
GAGCCGGTGA	GCGTGGGTCT	CGCGGTATCA	TTGCAGCACT	GGGGCCAGAT	950
GGTAAGCCCT	CCCGTATCGT	AGTTATCTAC	ACGACGGGGA	GTCAGGCAAC	1000
TATGGATGAA	CGAAATAGAC	AGATCGCTGA	GATAGGTGCC	TCACTGATTA	1050
AGCATTGGTA	ACTGTCAGAC	CAAGTTTACT	CATATATACT	TTAGATTGAT	1100
TTAAAACTTC	ATTTTTAATT	TAAAAGGATC	TAGGTGAAGA	TCCTTTTTGA	1150
TAATCTCATG	ACCAAAATCC	CTTAACGTGA	GTTTTCGTTC	CACTGAGCGT	1200
CAGACCCCGT	AGAAAAGATC	AAAGGATCTT	CTTGAGATCC	TTTTTTTCTG	1250
CGCGTAATCT	GCTGCTTGCA	AACAAAAAAA	CCACCGCTAC	CAGCGGTGGT	1300
TTGTTTGCCG	GATCAAGAGC	TACCAACTCT	TTTTCCGAAG	GTAACTGGCT	1350
TCAGCAGAGC	GCAGATACCA	AATACTGTCC	TTCTAGTGTA	GCCGTAGTTA	1400
GGCCACCACT	TCAAGAACTC	TGTAGCACCG	CCTACATACC	TCGCTCTGCT	1450
AATCCTGTTA	CCAGTGGCTG	CTGCCAGTGG	CGATAAGTCG	TGTCTTACCG	1500
GGTTGGACTC	AAGACGATAG	TTACCGGATA	AGGCGCAGCG	GTCGGGCTGA	1550
ACGGGGGGTT	CGTGCACACA	GCCCAGCTTG	GAGCGAACGA	CCTACACCGA	1600
ACTGAGATAC	CTACAGCGTG	AGCATTGAGA	AAGCGCCACG	CTTCCCGAAG	1650
GGAGAAAGGC	GGACAGGTAT	CCGGTAAGCG	GCAGGGTCGG	AACAGGAGAG	1700
CGCACGAGGG	AGCTTCCAGG	GGGAAACGCC	TGGTATCTTT	ATAGTCCTGT	1750
CGGGTTTCGC	CACCTCTGAC	TTGAGCGTCG	ATTTTTGTGA	TGCTCGTCAG	1800

FIG. 6a

10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
GGGGGCGGAG	CCTATGGAAA	AACGCCAGCA	ACGCGGCCTT	TTTACGGTTC	1850
CTGGCCTTTT	GCTGGCCTTT	TGCTCACATG	TTCTTTCCTG	CGTTATCCCC	1900
TGATTCTGTG	GATAACCGTA	TTACCGCCTT	TGAGTGAGCT	GATACCGCTC	1950
GCCGCAGCCG	AACGACCGAG	CGCAGCGAGT	CAGTGAGCGA	GGAAGCGGAA	2000
GAGCGCCCAA	TACGCAAACC	GCCTCTCCCC	GCGCGTTGGC	CGATTCATTA	2050
ATGCAGCTGG	CACGACAGGT	TTCCCGACTG	GAAAGCGGGC	AGTGAGCGCA	2100
ACGCAATTAA	TGTGAGTTAG	CTCACTCATT	AGGCACCCCA	GGCTTTACAC	2150
TTTATGCTTC	CGGCTCGTAT	GTTGTGTGGA	ATTGTGAGCG	GATAACAATT	2200
TCACACAGGA	AACAGCTATG	ACCATGATTA	CGAATTCGAG	CTCGGTACCC	2250
GGGGATCCTC	TAGAGTCGAC	CTGCAGGCAT	GCAAGCTTGC	ATGCCTGCAG	2300
GTCGACGCTC	GCGCGACTTG	GTTTGCCATT	CTTTAGCGCG	CGTCGCGTCA	2350
CACAGCTTGG	CCACAATGTG	GTTTTTGTCA	AACGAAGATT	CTATGACGTG	2400
TTTAAAGTTT	AGGTCGAGTA	AAGCGCAAAT	CTTTTTTAAC	CCTAGAAAGA	2450
TAGTCTGCGT	AAAATTGACG	CATGCATTCT	TGAAATATTG	CTCTCTCTTT	2500
CTAAATAGCG	CGAATCCGTC	GCTGTGCATT	TAGGACATCT	CAGTCGCCGC	2550
TTGGAGCTCC	CGTGAGGCGT	GCTTGTCAAT	GCGGTAAGTG	TCACTGATTT	2600
TGAACTATAA	CGACCGCGTG	AGTCAAAATG	ACGCATGATT	ATCTTTTACG	2650
TGACTTTTAA	GATTTAACTC	ATACGATAAT	TATATTGTTA	TTTCATGTTC	2700
TACTTACGTG	ATAACTTATT	ATATATATAT	TTTCTTGTTA	TAGATATCGT	2750
GACTAATATA	TAATAAAATG	GGTAGTTCTT	TAGACGATGA	GCATATCCTC	2800
TCTGCTCTTC	TGCAAAGCGA	TGACGAGCTT	GTTGGTGAGG	ATTCTGACAG	2850
TGAAATATCA	GATCACGTAA	GTGAAGATGA	CGTCCAGAGC	GATACAGAAG	2900
AAGCGTTTAT	AGATGAGGTA	CATGAAGTGC	AGCCAACGTC	AAGCGGTAGT	2950
GAAATATTAG	ACGAACAAAA	TGTTATTGAA	CAACCAGGTT	CTTCATTGGC	3000
TTCTAACAGA	ATCTTGACCT	TGCCACAGAG	GACTATTAGA	GGTAAGAATA	3050
AACATTGTTG	GTCAACTTCA	AAGTCCACGA	GGCGTAGCCG	AGTCTCTGCA	3100
CTGAACATTG	TCAGATCTCG	AGCTCAAGCT	TCGAATTCTG	CAGTCGACGG	3150
TACCCGATCT	TGTCGCCCGA	ACGCAGCGAC	AGAGATTCCA	ATGTGTCCGT	3200
ATCTTTCAGG	CTTTTGCCCT	TCAGTTCCAG	ACGAAGCGAC	TGGCGATTCG	3250
CGTGTGGGGT	CTGCTTCAGG	GTCTTGTGAA	TTAGGCGCCG	CAGATCGCCG	3300
ATGGGCGTGG	CGCCGGAGGG	CACCTTCACC	TTGCCGTACG	GCTTGCTGTT	3350
CTTCGCGTTC	AAAATCTCCA	GCTCCATTTT	GCTTTCGGTG	CGCTTGCAAT	3400
CAGTACTGTC	CAAAATCGAA	AATCGCCGAA	CCGTAGTGTG	ACCGTGCGGG	3450
GCTCTGCGAA	AATAAACTTT	TTTAGGTATA	TGGCCACACA	CGGGGAAAGC	3500
ACAGTGGATT	ATATGTTTTA	ATATTATAAT	ATGCAGGTTT	TCATTACTTA	3550
TCCAGATGTA	AGCCCACTTA	AAGCGATTTA	ACAATTATTT	GCCGAAAGAG	3600

FIG. 6b

10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
TAAAAACAAA	TTTCACTTAA	AAATGGATTA	AGAAAAGCTT	GTGTAAGATT	3650
ATGCGCAGCG	TTGCCAGATA	GCTCCATTTA	AAACACTTCA	AAAACAATAA	3700
GTTTTGAAAA	TATATACATA	AATAGCAGTC	GTTGCCGCAA	CGCTCAACAC	3750
ATCACACTTT	TAAAACACCC	TTTACCTACA	CAGAATTACT	TTTTAAATTT	3800
CCAGTCAAGC	TGCGAGTTTC	AAAATTATAG	CCGGTAGAGA	AGACAGTGCT	3850
ATTTCAAAAG	CAAACTAAAT	AAACACCAAT	CCTAACAAGC	CTTGGACTTT	3900
TGTAAGTTTA	GATCAAAGGT	GGCATTGCAT	TCAATGTCAT	GGTAAGAAGT	3950
AGGTCGTCTA	GGTAGAAATC	CTCATTCAGC	CGGTCAAGTC	AGTACGAGAA	4000
AGGTCTCAAT	TTGAAATTGT	CTTAAAAATA	TTTTATTGTT	TTGTACTGTG	4050
GTGAGTTTAA	ACGAAAAACA	CAAAAAAAAA	GTGATACACA	GAAATCATAA	4100
AAAATTTTAA	TACAAGGTAT	TCGTACGTAT	CAAAAACATT	TCGGCACAAT	4150
TTTTTTTCTC	TGTACTAAAG	TGTTACGAAC	ACTACGGTAT	TTTTTAGTGA	4200
TTTTCAACGG	ACACCGAAGG	TATATAAACA	GCGTTCGCGA	ACGGTCGCCT	4250
TCAAAACCAA	TTGACATTTG	CAGCAGCAAG	TACAAGCAGA	AAGTAAAGCG	4300
CAATCAGCGA	AAAATTTATA	CTTAATTGTT	GGTGATTAAA	GTACAATTAA	4350
AAGAACATTC	TCGAAAGTCA	CAAGAAACGT	AAGTTTTTAA	CTCGCTGTTA	4400
CCAATTAGTA	ATAAGAGCAA	CAAGACGTTG	AGTAATTTCA	AGAAAAACTG	4450
CATTTCAAGG	TCTTTGTTCG	GCCATTTTTT	TTTTATTCAA	CGCTCTACGT	4500
AATTACAAAA	TAAGAAATTG	GCAGCCACGC	ATCTTGTTTT	CCCAATCAAT	4550
TGGCATCAAA	ACGCAAACAA	ATCTATAAAT	AAAACTTGCG	TGTTGATTTT	4600
CGCCAAGATT	TATTGGCAAA	TTGTGAAATT	CGCAGTGACG	CATTTGAAAA	4650
TTCGAGAAAT	CACGAACGCA	CTCGAGCATT	TGTGTGCATG	TTATTAGTTA	4700
GTTAGTTCTT	TGCTTAATTG	AAGTATTTTA	CCAACGAAAT	CCACTTATTT	4750
TTAGCTGAAA	TAGAGTAGGT	TGCTTGAAAC	GAAAGCCACG	TCTGGAAAAT	4800
TTCTTATTGC	TTAGTAGTTG	TGACGTCACC	ATATACACAC	AAAATAATGT	4850
GTTATGCATGC	GTTTCAGCTG	TGTATATATA	CATGCACACA	CTCGCATTAT	4900
GAAAACGATG	ACGAGCAACG	GAACAGGTTT	CTCAACTACC	TTTGTTCCTG	4950
TTTCTTCGCT	TTCCTTTGTT	CCAATATTCG	TAGAGGGTTA	ATAGGGGTTT	5000
CTCAACAAAG	TTGGCGTCGA	TAAATAAGTT	TCCCATTTTT	ATTCCCCAGC	5050
CAGGAAGTTA	GTTTCAATAG	TTTTGTAATT	TCAACGAAAC	TCATTTGATT	5100
TCGTACTAAT	TTTCCACATC	TCTATTTTGA	CCCGCAGAAT	AATCCAAAAT	5150
GCAGATCGGG	GATCCCACCC	CACCCAAGAA	GAAGCGCAAG	GTGGAGGACG	5200
ATCCCGTCGT	TTTACAACGT	CGTGACTGGG	AAAACCCTGG	CGTTACCCAA	5250
CTTAATCGCC	TTGCAGCACA	TCCCCCTTTC	GCCAGCTGGC	GTAATAGCGA	5300
AGAGGCCCGC	ACCGATCGCC	CTTCCCAACA	GTTGCGGTCG	ACTCTAGAGG	5350
ATCCCCGGGA	TCCACCGGTC	GCCACCATGG	TGAGCAAGGG	CGAGGAGCTG	5400

FIG. 6 c

10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
TTCACCGGGG	TGGTGCCCAT	CCTGGTCGAG	CTGGACGGCG	ACGTAAACGG	5450
CCACAAGTTC	AGCGTGTCCG	GCGAGGGCGA	GGGCGATGCC	ACCTACGGCA	5500
AGCTGACCCT	GAAGTTCATC	TGCACCACCG	GCAAGCTGCC	CGTGCCCTGG	5550
CCCACCCTCG	TGACCACCCT	GACCTACGGC	GTGCAGTGCT	TCAGCCGCTA	5600
CCCCGACCAC	ATGAAGCAGC	ACGACTTCTT	CAAGTCCGCC	ATGCCCGAAG	5650
GCTACGTCCA	GGAGCGCACC	ATCTTCTTCA	AGGACGACGG	CAACTACAAG	5700
ACCCGCGCCG	AGGTGAAGTT	CGAGGGCGAC	ACCCTGGTGA	ACCGCATCGA	5750
GCTGAAGGGC	ATCGACTTCA	AGGAGGACGG	CAACATCCTG	GGGCACAAGC	5800
TGGAGTACAA	CTACAACAGC	CACAACGTCT	ATATCATGGC	CGACAAGCAG	5850
AAGAACGGCA	TCAAGGTGAA	CTTCAAGATC	CGCCACAACA	TCGAGGACGG	5900
CAGCGTGCAG	CTCGCCGACC	ACTACCAGCA	GAACACCCCC	ATCGGCGACG	5950
GCCCCGTGCT	GCTGCCCGAC	AACCACTACC	TGAGCACCCA	GTCCGCCCTG	6000
AGCAAAGACC	CCAACGAGAA	GCGCGATCAC	ATGGTCCTGC	TGGAGTTCGT	6050
GACCGCCGCC	GGGATCACTC	TCGGCATGGA	CGAGCTGTAC	AAGTAAAGCG	6100
GCCGCGACTC	TAGATCATAA	TCAGCCATAC	CACATTTGTA	GAGGTTTTAC	6150
TTGCTTTAAA	AAACCTCCCA	CACCTCCCCC	TGAACCTGAA	ACATAAAATG	6200
AATGCAATTG	TTGTTGTTAA	CTTGTTTATT	GCAGCTTATA	ATGGTTACAA	6250
ATAAAGCAAT	AGCATCACAA	ATTTCACAAA	TAAAGCATTT	TTTTCACTGC	6300
ATTCTAGTTG	TGGTTTGTCC	AAACTCATCA	ATGTATCTTA	AGGCGTAAAT	6350
TGTAAGCGTT	AATATTTTGT	TAAAATTCGC	GTTAAATTTT	TGTTAAATCA	6400
GCTCATTTTT	TAACCAATAG	GCCGAAATCG	GCAAAATCCC	TTATAAATCA	6450
AAAGAATAGA	CCGAGATAGG	GTTGAGTGTT	GTTCCAGTTT	GGAACAAGAG	6500
TCCACTATTA	AAGAACGTGG	ACTCCAACGT	CAAAGGGCGA	AAAACCGTCT	6550
ATCAGGGCGA	TGGCCCACTA	CGTGAACCAT	CACCCTAATC	AAGTTTTTTG	6600
GGGTCGAGGT	GCCGTAAAGC	ACTAAATCGG	AACCCTAAAG	GGAGCCCCCG	6650
ATTTAGAGCT	TGACGGGGAA	AGCCGGCGAA	CGTGGCGAGA	AAGGAAGGGA	6700
AGAAAGCGAA	AGGAGCGGGC	GCTAGGGCGC	TGGCAAGTGT	AGCGGTCACG	6750
CTGCGCGTAA	CCACCACACC	CGCCGCGCTT	AATGCGCCGC	TACAGGGCGC	6800
GTCAGGTGGC	ACTTTTCGGG	GAAATGTGCG	CGGAACCCCT	ATTTGTTTAT	6850
TTTTCTAAAT	ACATTCAAAT	ATGTATCCGC	TCATGAGACA	ATAACCCTGA	6900
TAAATGCTTC	AATAATATTG	AAAAAGGAAG	AGTCCTGAGG	CGGAAAGAAC	6950
CAGCTGTGGA	ATGTGTGTCA	GTTAGGGTGT	GGAAAGTCCC	CAGGCTCCCC	7000
AGCAGGCAGA	AGTATGCAAA	GCATGCATCT	CAATTAGTCA	GCAACCAGGT	7050
GTGGAAAGTC	CCCAGGCTCC	CCAGCAGGCA	GAAGTATGCA	AAGCATGCAT	7100
CTCAATTAGT	CAGCAACCAT	AGTCCCGCCC	CTAACTCCGC	CCATCCCGCC	7150
CCTAACTCCG	CCCAGTTCCG	CCCATTCTCC	GCCCCATGGC	TGACTAATTT	7200

FIG. 6d

10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
TTTTTATTTA	TGCAGAGGCC	GAGGCCGCCT	CGGCCTCTGA	GCTATTCCAG	7250
AAGTAGTGAG	GAGGCTTTTT	TGGAGGAACC	ATTGTGGGAA	CCGTGCGATC	7300
AAACAAACGC	GAGATACCGG	AAGTACTGAA	AAACAGTCGC	TCCAGGCCAG	7350
TGGGAACATC	GATGTTTTGT	TTTGACGGAC	CCCTTACTCT	CGTCTCATAT	7400
AAACCGAAGC	CAGCTAAGAT	GGTATACTTA	TTATCATCTT	GTGATGAGGA	7450
TGCTTCTATC	AACGAAAGTA	CCGGTAAACC	GCAAATGGTT	ATGTATTATA	7500
ATCAAACTAA	AGGCGGAGTG	GACACGCTAG	ACCAAATGTG	TTCTGTGATG	7550
ACCTGCAGTA	GGAAGACGAA	TAGGTGGCCT	ATGGCATTAT	TGTACGGAAT	7600
GATAAACATT	GCCTGCATAA	ATTCTTTTAT	TATATACAGC	CATAATGTCA	7650
GTAGCAAGGG	AGAAAAGGTC	CAAAGTCGCA	AAAAATTTAT	GAGAAACCTT	7700
TACATGAGCC	TGACGTCATC	GTTTATGCGT	AAGCGTTTAG	AAGCTCCTAC	7750
TTTGAAGAGA	TATTTGCGCG	ATAATATCTC	TAATATTTTG	CCAAATGAAG	7800
TGCCTGGTAC	ATCAGATGAC	AGTACTGAAG	AGCCAGTAAT	GAAAAAACGT	7850
ACTTACTGTA	CTTACTGCCC	CTCTAAAATA	AGGCGAAAGG	CAAATGCATC	7900
GTGCAAAAAA	TGCAAAAAAG	TTATTTGTCG	AGAGCATAAT	ATTGATATGT	7950
GCCAAAGTTG	TTTCTGACTG	ACTAATAAGT	ATAATTTGTT	TCTATTATGT	8000
ATAAGTTAAG	CTAATTACTT	ATTTTATAAT	ACAACATGAC	TGTTTTTAAA	8050
GTACAAAATA	AGTTTATTTT	TGTAAAAGAG	AGAATGTTTA	AAAGTTTTGT	8100
TACTTTATAG	AAGAAATTTT	GAGTTTTTGT	TTTTTTTTAA	TAAATAAATA	8150
AACATAAATA	AATTGTTTGT	TGAATTTATT	ATTAGTATGT	AAGTGTAAAT	8200
ATAATAAAAC	TTAATATCTA	TTCAAATTAA	TAAATAAACC	TCGATATACA	8250
GACCGATAAA	ACACATGCGT	CAATTTTACG	CATGATTATC	TTTAACGTAC	8300
GTCACAATAT	GATTATCTTT	CTAGGGTTAA	ATAATAGTTT	CTAATTTTTT	8350
TATTATTCAG	CCTGCTGTCG	TGAATACCGT	ATATCTCAAC	GCTGTCTGTG	8400
AGATTGTCGT	ATTCTAGCCT	TTTTAGTTTT	TCGCTCATCG	ACTTGATATT	8450
GTCCGACACA	TTTTCGTCGA	TTTGCGTTTT	GATCAAAGAC	TTGAGCAGAG	8500
ACACGTTAAT	CAACTGTTCA	AATTGATCCA	TATTAACGAT	ATCAACCCGA	8550
TGCGTATATG	GTGCGTAAAA	TATATTTTTT	AACCCTCTTA	TACTTTGCAC	8600
TCTTGCGTTAA	TACGCGTTCG	TGTACAGACG	TAATCATGTT	TTCTTTTTTG	8650
GATTAAAACTC	CTACTGAGTT	TGACCTCATA	TTAGACCCTC	ACAAGTTGCA	8700
AAACGTGGCA	TTTTTTACCA	ATGAAGAATT	TAAAGTTATT	TTAAAAAATT	8750
TCATTCACAGA	TTTAAAGAAG	AACCAAAAAT	TAAATTATTT	CAACAGTTTA	8800
ATCCGACCAGT	TAATCAACGT	GTACACAGAC	GCGTCGGCAA	AAAACACGCA	8850
GCCCGACGTG	TTGCCTAAAA	TTATTAAATC	AACTTGTGTT	ATAGTCACGG	8900
ATTTTGCCGTC	CAACGTGTTC	CTCAAAAAGT	TGAAGACCAA	CAAGTTTACG	8950
GACCACTATTA	ATTATTTGAT	TTTGCCCCAC	TTCATTTTGT	GGGATCACAA	9000

FIG. 6e

10	20	30	40	50	
1234567890	1234567890	1234567890	1234567890	1234567890	
TTTGTTATA	TTTAAACAA	AGCTTGGCAC	TGGCCGTCGT	TTTACAACGT	9050
CGTGACTGGG	AAAACCCTGG	CGTTACCCAA	CTTAATCGCC	TTGCAGCACA	9100
TCCCCCTTTC	GCCAGCTGGC	GTAATAGCGA	AGAGGCCCGC	ACCGATCGCC	9150
CTTCCCAACA	GTTGCGCAGC	CTGAATGGCG	AATGGCGCCT	GATGCGGTAT	9200
TTTCTCCTTA	CGCATCTGTG	CGGTATTTCA	CACCGCATAT	GGTGCACTCT	9250
CAGTACAATC	TGCTCTGATG	CCGCATAGTT	AAGCCAGCCC	CGACACCCGC	9300
CAACACCCGC	TGACGCGCCC	TGACGGGCTT	GTCTGCTCCC	GGCATCCGCT	9350
TACAGACAAG	CTGTGACCGT	CTCCGGGAGC	TGCATGTGTC	AGAGGTTTTC	9400
ACCGTCATCA	CCGAAACGCG	CGA			9423

FIG. 6 f

FIG. 7a

FIG. 7b

FIG. 7c

FIG. 7d

FIG. 7e

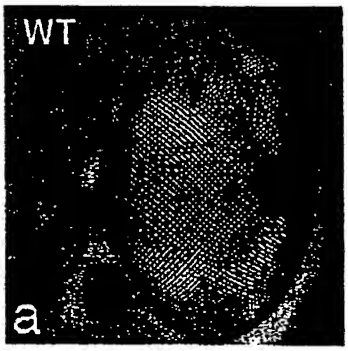

FIG. 8a

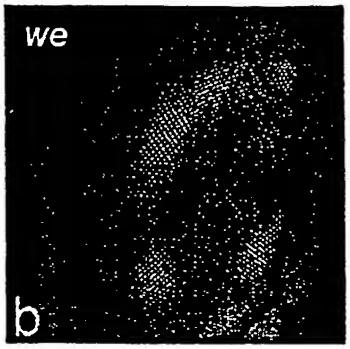

FIG. 8b

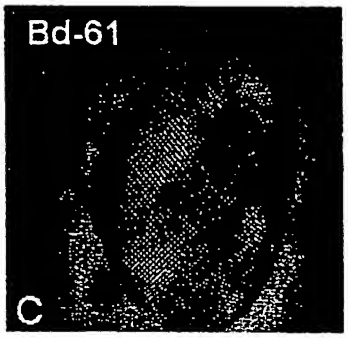

FIG. 8c

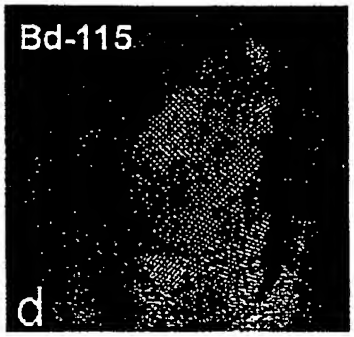

FIG. 8d

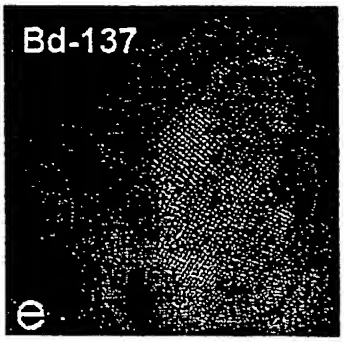

FIG. 8e

FIG. 9a

FIG. 9b

FIG. 10

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tctaaataca ttcaaatatg tatccgctca tgagacaata accctgataa atgcttcaat 180

aatattgaaa aaggaagagt atgagtattc aacatttccg tgtcgccctt attccctttt 240

ttgcggcatt ttgccttcct gtttttgctc acccagaaac gctggtgaaa gtaaaagatg 300
ctgaagatca gttgggtgca cgagtgggtt acatcgaact ggatctcaac agcggtaaga 360

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tatgtggcgc ggtattatcc cgtattgacg ccgggcaaga gcaactcggt cgccgcatac 480

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gcatgacagt aagagaatta tgcagtgctg ccataaccat gagtgataac actgcggcca 600

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gggatcatgt aactcgcctt gatcgttggg aaccggagct gaatgaagcc ataccaaacg 720

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/22433

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12N 15/00, 15/63; A01K 67/00, 67/027
US CL :435/320.1, 455; 800/13, 14, 19, 20

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/320.1, 455; 800/13, 14, 19, 20

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST, STN, MEDLINE, CAPLUS, BIOSIS, SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HANDLER et al., The Lepidopteran Transposon Vector, piggybac, Mediates germ-Line Transformation in the Mediterranean Fruit Fly. Proc. Natl. Acad. Sci., USA. June 1998, Vol. 95, pp. 7520-7525, especially pages 7520, 7521.	1-9
Y	CORMACK et al. ACS-Optimized Mutants of the Green Fluorescent Protein (GFP). Science. 1996, Vol. 173, pp. 33-38, especially pages, 33, 38.	1-9
Y	DAVIS et al. A Nuclear GFP That Marks Nuclei in Living Drosophila Embryos; Maternal Supply Overcomes a Delay in the Appearance of Zygotic Fluorescence. Developmental Biology. 1995, Vol. 170, pp. 726-729, especially pages, 726, 729.	1-9

[X] Further documents are listed in the continuation of Box C. . . [ ] . . . See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
22 SEPTEMBER 2000	02 NOV 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer SHIN-LIN CHEN
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US00/22433

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	O'BROCHTA et al. Transposable Elements and Gene Transformation in Non-Drosophilid Insects, Insect Biochem. Molec. Biol., 1996, Vol. 26, Nos. 8-9, pp. 739-753, especially pages 739, 740, 749, 750.	7-9
A	ASHBURNER et al. Prospects for the Genetic Transformation of Arthropods. Insect Molecular Biology. 1998, Vol. 7, No. 3, pp. 201-213, especially pages 201, 205.	7-9